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Functional Organization of Saposin C

DEFINITION OF THE NEUROTROPHIC AND ACID β-GLUCOSIDASE ACTIVATION REGIONS*

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Saposin C is an essential co-factor for the hydrolysis of glucosylceramide by acid β-glucosidase in mammals. In addition, prosaposin promotes neurite outgrowth *in vitro* via sequences in saposin C. The regional organization of these neurotrophic and activation properties of saposin C was elucidated using recombinant or chemically synthesized saposin Cs from various regions of the molecule. Unreduced and reduced proteins were analyzed by electrospray-mass spectrometry to establish the complement of disulfide bonds in selected saposin Cs. Using saposin B as a unreactive backbone, chimeric saposins containing various length segments of saposin B and C localized the neurotrophic and acid β-glucosidase activation properties to the carboxyl- and NH_2-terminal 50% of saposin C, respectively. The peptide spanning residues 22–31 had neurotrophic effects. Molecular modeling and site-directed mutagenesis localized the activation properties of saposin C to the region spanning residues 47–62. Secondary structure was needed for retention of this property. Single substitutions of R and S at the conserved cysteines at 47 or 78 diminished but did not obliterate the activation properties. These results indicate the segregation of neurotrophic and activation properties of saposin C to two different faces of the molecule and suggest a topographic sequestration of the activation region of prosaposin for protection of the cell from adverse hydrolytic activity of acid β-glucosidase.

The *in vivo* activities of several lysosomal hydrolases require the presence of small (~80 amino acids) heat stable activator proteins or saposins (1–3). Saposin C was first described by Ho and O'Brien (4) in 1971 as a soluble factor that stimulated the activity of acid β-glucosidase (EC 3.2.1.45, *N*-acyl-sphingosyl-β-D-glucoside:glucohydrolase). This saposin and three other highly homologous saposins, A, B and D, are derived from the same precursor, prosaposin, by proteolytic processing (5–8). Saposins A, B, C, and D are encoded in the same reading frame of a primary transcript (see Fig. 1*A*) (5–7). In addition to their highly similar sequences (~50% amino acid identity), each saposin has six strictly conserved cysteines (see Fig. 1*B*). For each saposin the primary amino acid sequences have been determined chemically and predicted from the cDNA (5–7, 9). Recently, the disulfide structures of saposins B and C have been solved (10), and those for saposins A and D are likely identical.

Saposin B functions as a physiological solubilizer of the glycosphingolipid substrates for arylsulfatase A (sulfatide, *in vivo*), α-galactosidase A (globotriaosylceramide, *in vitro*), and β-galactosidase (G_{M1}-ganglioside, *in vitro*) (2). The affinities of these substrates for saposin B apparently forms the basis for its differential metabolic effects, and a general model has been proposed for its mechanism of action (2). The physiologic functions of saposins A and D are not defined, although *in vitro* and *ex vivo* studies show that saposin D enhances ceramidase activity (11).

Although saposin C is structurally similar to saposin B, the saposin C mechanism of action is different and less completely defined. Saposin C binds to and may transfer gangliosides between artificial membranes (12), but it does not bind glucosylceramide, the acid β-glucosidase substrate (13). Saposin C binds directly to acid β-glucosidase (13) and may alter the structure of negatively charged phospholipid, particularly phosphatidylserine, interfaces (10). These function to conform acid β-glucosidase and possibly alter binding at the lipid interface to effect an enhancement of substrate hydrolytic rates (13, 14). Interaction of saposin C with acid β-glucosidase is complex and involves high (specific) and low affinity sites (15). The latter may interfere with a binding site on the enzyme for phosphatidylserine, leading to diminished activation (13, 15). Recently, recombinant (15) and chemically synthesized (16) peptides were used to regionally localize the activation and binding components of saposin C to its carboxyl-terminal 50%.

During the conduct of these studies, prosaposin was shown to have glycosphingolipid binding and transport properties (12) and neurotrophic effects *in vitro* (17). The neurite outgrowth properties of prosaposin appear to regionally localize to the NH_2-terminal 50% of saposin C (18). Consequently, the biological roles of prosaposin may be multidimensional and include plasma membrane-mediated signal transduction events as well as essential intracellular glucosylceramide catabolic regulation. In this communication recombinant and chemically synthesized saposin Cs are used to explore the functional segregation of its neurotrophic and activation properties. A variety of chimeric and mutagenized saposin Cs were created to characterize residues critical to its neurotrophic effects and its interaction with acid β-glucosidase.

EXPERIMENTAL PROCEDURES

Materials—The following were from commercial sources: 4-methylumbelliferyl-β-D-glucopyranoside and Ceredase® (Genzyme Corp., Boston, MA); phosphatidylserine (Avanti, Alabaster, AL); Sequenase® and Sequenase kit (U. S. Biochemical Corp., Cleveland, OH); restriction endonucleases (New England Biolabs, Beverly, MA); oligonucleotide synthesis and PhastSystem reagents (Pharmacia Biotech Inc.); pET

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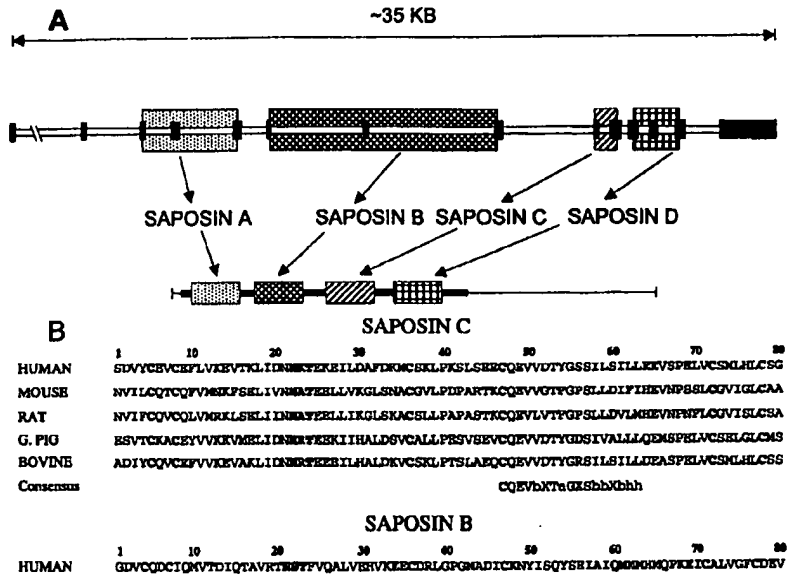

FIG. 1. **Schematic diagram (*A*) and sequences (*B*) of the prosaposin gene, cDNA, and amino acid residues.** In *A* the genomic human DNA including the promoter (Y. Sun and G. A. Grabowski, unpublished observations) occupies about 35 kilobase pairs on chromosome 10q. The *black rectangles* represent the 14 exons, and the *white horizontal bars* are the intronic sequences. The initiating ATG is in the first exon. The saposin A, B, C, and D regions are indicated by similar shadings of regions from the gene and cDNA. The mouse genomic organization is essentially identical (Y. Sun and G. A. Grabowski, unpublished observations). In *B* is shown the saposin C amino acid sequence homology between several species. A derived consensus sequence for residues 47–63 is shown: *h*, hydrophobic amino acid; *b*, branch chain amino acid; *a*, aromatic amino acid; *X*, any amino acid. Placement of all cysteines and the *N*-glycosylation sites are conserved. The amino acid sequence of human saposin B is shown *at the bottom*.

21-a(+) DNA vector, *Escherichia coli* host strain [BL21 (DE3)] and His·Bind resin (Novagen, Madison, WI); Magic® polymerase chain reaction prep kits and Magic® Minipreps kit (Promega, Madison, WI); C_4-reverse phase Delta-pak 300A, 5 μm, 3.9×150-mm HPLC[1] column (Waters Chromatography, Milford, MA); ciliary neurotrophic factor (R & D Systems, Minneapolis, MN); Dibutyryl-cAMP (Sigma); synthesized saposin peptides (N22-A31, Q48-G55, and T53-L62; >98% purity), human angiotensin I, II, and III, and bradykinin (Penisula Laboratories, Inc., Belmont, CA). Cell line Neuro2A was from Dr. Richard Ackeson (Cincinnati, OH), and NS20Y was from Dr. Marshall Nirenberg (National Institutes of Health). Organic solvents were HPLC grade, and other chemicals were reagent grade or better. The full-length mouse prosaposin cDNA was cloned from a λZap library (Stratagene, La Jolla, CA) and fully characterized by sequence analysis. It was identical to those previously reported (9).

Mutagenesis, Expression, and Purification of Saposins and Saposin Fragments—Oligonucleotides were synthesized with a Gene Assembler 4 (Pharmacia) and used without purification. The normal and mutant saposins and fragments are shown in Fig. 2 (*A* and *B*). The mature NH_2-terminal amino acid from the natural saposin C or B will be designated 1 for numbering purposes to identify the inclusive amino acids in a fragment (Figs. 1 and 2). For the chimeric saposins the amino acid numbers will correspond to those in the original saposin, *e.g.* in the saposin containing the NH_2-terminal 50% of saposin B, residues 1–41, and the carboxyl-terminal 50% of saposin C, residues 42–80, the saposin B or C amino acids will be those from the cognate saposin; *i.e.* saposin B(1–41)C(42–80). The DNAs for the chimeric saposins composed of human saposins B and C or human and mouse saposin C were synthesized using polymerase chain reaction. The constructs were cloned into *Bam*HI–*Hin*dIII or *Bam*HI–*Eco*RI sites of pET 21-a(+). Single or multiple point mutations were introduced into saposin C by polymerase chain reaction in the presence of a mutagenic oligonucleotide and cloned into the *Bam*HI–*Hin*dIII or *Bam*HI–*Bsi*WI sites of pET 21-a(+). The fidelity of all saposin constructs was verified by sequence analyses. Those saposin C constructs expressed by pET 21-a(+) contained the nonsaposin [pET 21-a(+)] amino acids MASMTGGQQMGRGS (NH_2-terminal) and KLAAALEHHHHHH (carboxyl-terminal). For saposin B the sequence was the same on the NH_2 terminus and was KENSSSVDKLAAALEHHHHHH on the carboxyl end. These have been shown not to alter saposin function (15). NH_2-terminal sequencing showed the absence of the initiating Met as expected because the immediate downstream amino acid is an alanine. In *E. coli* the presence of this Ala signals the cleavage of the preceding Met (19, 20).

Expression of the saposins in *E. coli* followed transformation into BL21(DE3) cells that contain a isopropyl-1-thio-β-D-galactopyranoside inducible T7-polymerase gene (15). Transformations, incubations, inductions with 1 mM isopropyl-1-thio-β-D-galactopyranoside, cell harvest, and isolation of pure proteins were as described previously (15). Natural saposin C was purified from Gaucher disease spleen as described (15).

HPLC (C_4 column) purification of the saposins was in 0.05% trifluoroacetic acid with an acetonitrile gradient (0–40% in 5 min and then 40–75% in 35 min) and were monitored by UV detection at 225 nm. The major peaks were collected and dried by rotatory evaporation and subjected to NH_2-terminal amino acid analysis by Edman degradation and/or mass determination by electrospray-mass spectrometry (ES-MS) (PE Sciex API-III, PE Sciex, Thornhill, Canada). A delivery solvent of a methanol/water (1:1, v/v) with 0.1% formic acid was used for flow injection experiments at a flow rate of 5 μl/min. Dried proteins were reduced by dissolving with 6 M guanidine HCl in 50 mM Tris-HCl, pH 8.0, (0.2 ml) with dithiothreitol (1 M, 5 μl) for 3 h at 37 °C. The proteins were reisolated by HPLC as above. The expected molecular weights were calculated (DNAsis, Hitachi Software Engineering, San Bruno, CA) from the predicted amino acid sequence of the fully reduced normal or mutant saposin including the flanking vector derived NH_2-terminal amino acids and the carboxyl-terminal His-tag (see above).

Estimation of Enzyme Activity—Acid β-glucosidase activities in the presence of phosphatidylserine and saposins were estimated by quantitating the fluorescent intensity of liberated 4-methylumbelliferone from the fluorogenic substrate, 4-methylumbelliferyl-β-D-glucopyranoside (15, 21). The usual reaction mixture (200 μl) contained 2 nM acid β-glucosidase, phosphatidylserine (0.4 μg/ml), 4-methylumbelliferyl-β-D-glucopyranoside (4 mM), 0.1 M sodium acetate, pH 4.7, and the indicated amounts of saposin (15). AC_{50} is used to indicate the activator concentration required to achieve 50% of maximal activity. Reactions were for 15–30 min (37 °C) and were terminated by the addition of 2.35 ml of 0.1 M ethylene diamine. Protein concentrations were estimated using bovine serum albumin as a standard (22).

Neurite Outgrowth Assays—Neuro2A and NS20Y cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37 °C. Neuro2A or NS20Y cells (4×10^4) during exponential growth phase were distributed in 35-mm polystyrene dishes and incubated for 24 h at which time the medium was replaced with fresh medium containing 0.5% fetal calf serum with or without added saposin peptides. After 24 h, the cells were rinsed with phosphate-buffered saline and then fixed in phosphate-buffered saline containing 4% paraformaldehyde. The number of cells bearing neurites were counted in several randomly chosen fields (200×). Neurites were identified by the presence of growth cones. Cells bearing neurites longer than 1.0× the cell diameter were scored as positive. Positive controls included ciliary neurotrophic factor (2.2 nM) for NS20Y cells and dibutyryl-cAMP for Neuro2A cells (23). The results are expressed as the mean and standard error from over 200 cells in each of at least three independent experiments. *t* tests were performed using Statview (Abacus Concepts Inc, Berkeley, CA).

[1] The abbreviations used are: HPLC, high pressure liquid chromatograph; ES-MS, electrospray-mass spectrometry.

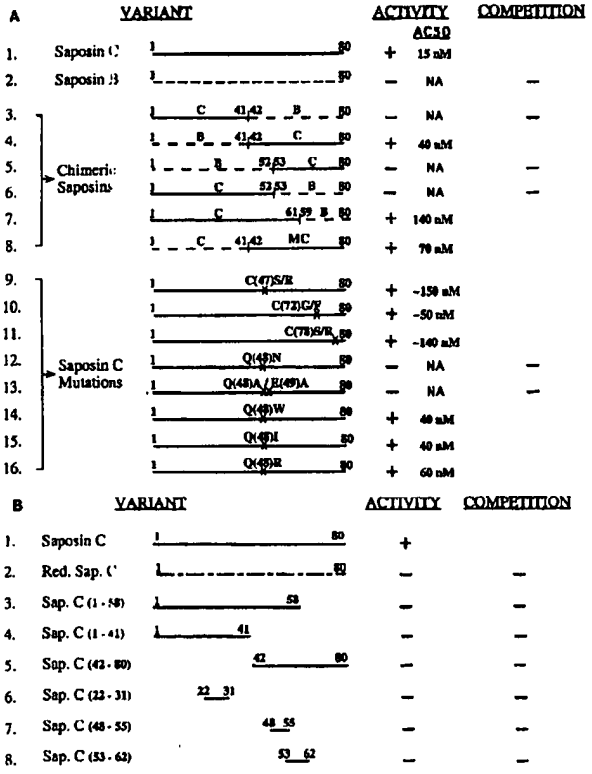

FIG. 2. **Schematic representation of the various saposin C fragments.** The mature saposin C NH_2-terminal amino acid (see Fig. 1*B*) is designated 1. The nomenclature used is as in the following example: saposin C(1–41)B(42–80) means that the chimeric saposin contains residues 1–41 from saposin C and residues 42–80 from saposin B. *MC* refers to the mouse saposin C. The AC_{50} value refers to the concentration of saposin required to achieve 50% of maximal acid β-glucosidase activation. The competition column refers to the ability of saposins that do not activate acid β-glucosidase to decrease the activity of the enzyme in the presence of 100 nM wild-type saposin C (15). A minus sign means that no effect was observed up to 500 nM. The blanks indicate those saposin Cs that activated the enzyme and were not tested as competitors. *A* depicts saposins that maintain the complete sequence length of saposin C that were chimeric (*rows 3–8*) or point-mutated (*rows 9–16*). *B* shows fragments that were synthesized recombinantly (*rows 1–5*) or chemically (*rows 6–8*). *Red. Sap. C* refers to the reduced and carboxymethylated saposin C.

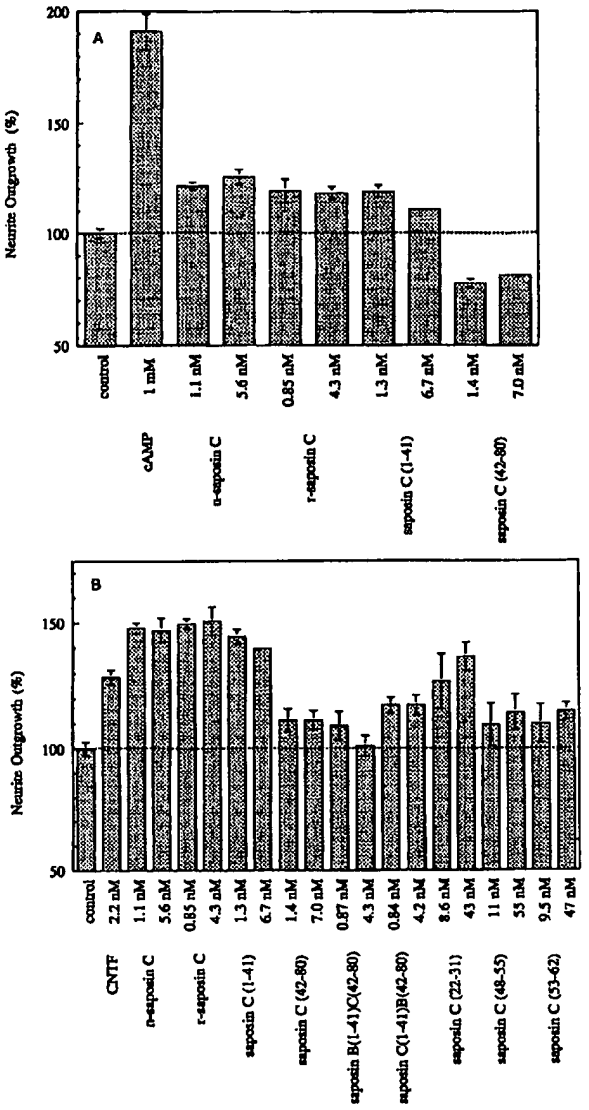

FIG. 3. **Neurite outgrowth effects of saposin C derivatives on Neuro2A (*A*) and NS20Y (*B*) cells.** 1 mM dibutyryl-cAMP (*A*) and 2.2 nM ciliary neurotrophic factor (*B*, *CNTF*) were used as controls for the positive effects in Neuro2A and NS20Y, respectively. The mean and standard errors are based on results from three separate experiments, each with over 200 cells counted. The absence of error bars means that the error was too small to display. The concentrations and saposin derivatives are shown below. *n-Saposin C* and *r-saposin C* refer to the natural and the recombinant saposin Cs, respectively.

RESULTS

Functional Segregation Neurotrophic and Acid β-Glucosidase Activation Effects of Saposin C—Natural splenic and recombinant saposin Cs at equimolar concentrations (~1–6 nM) similarly promoted neurite outgrowth in Neuro2A (Fig. 3*A*) ($p > 0.64$) and NS20Y cells (Fig. 3*B*) ($p > 0.5$). The neurite outgrowth was much greater with NS20Y cells. Recombinant saposin C(1–41) (Fig. 3*B*) had similar effects, whereas saposin C(42–80) had no major effect ($p > 0.2$). In Neuro2A cells saposin C(42–80) reproducibly inhibited neurite outgrowth (Fig. 3*A*) ($p < 0.01$). Neurite outgrowth effects of saposin C(1–41) and saposin C(1–41)B(42–80) were similar in NS20Y cells (Fig. 3*B*). Saposin B(1–41)C(42–80) has no major effect on neurite outgrowth ($p = 0.9$). Chemically synthesized saposin C(22–31) has about 50–70% of the neurite effect of the saposin C(1–41) (Fig. 3*B*) and about 30% greater effects at 43 nM compared with 8.6 nM ($p = 0.015$). These amounts were 1–6 times more than those used for saposin C or saposin C(1–41). Saposin C(48–55) or C(53–63) at ~40 nM had little effect compared with saposin C(22–31) ($p < 0.008$).

In comparison, the acid β-glucosidase activation effect was mediated by the carboxyl-terminal 50% of saposin C (Figs. 2 and 4). The chimeric saposin B(1–41)C(42–80) had ~70% of the activation effect of wild-type saposin C. Saposin C(1–41)B(42–80) was not active (Fig. 4*A*). Saposin C(1–41), C(1–58) and saposin C(42–80) were inactive (Fig. 2). These above results show that the neurotrophic and acid β-glucosidase activation properties of saposin C are segregate to the NH_2- and carboxyl-terminal 50% of the molecule, respectively. Also, a linear peptide of residues 22–31 is sufficient for neurotrophic effects, but higher order structures in the carboxyl-terminal 50% are needed for acid β-glucosidase activation effects.

Analysis of Acid β-Glucosidase Activation by Saposin C

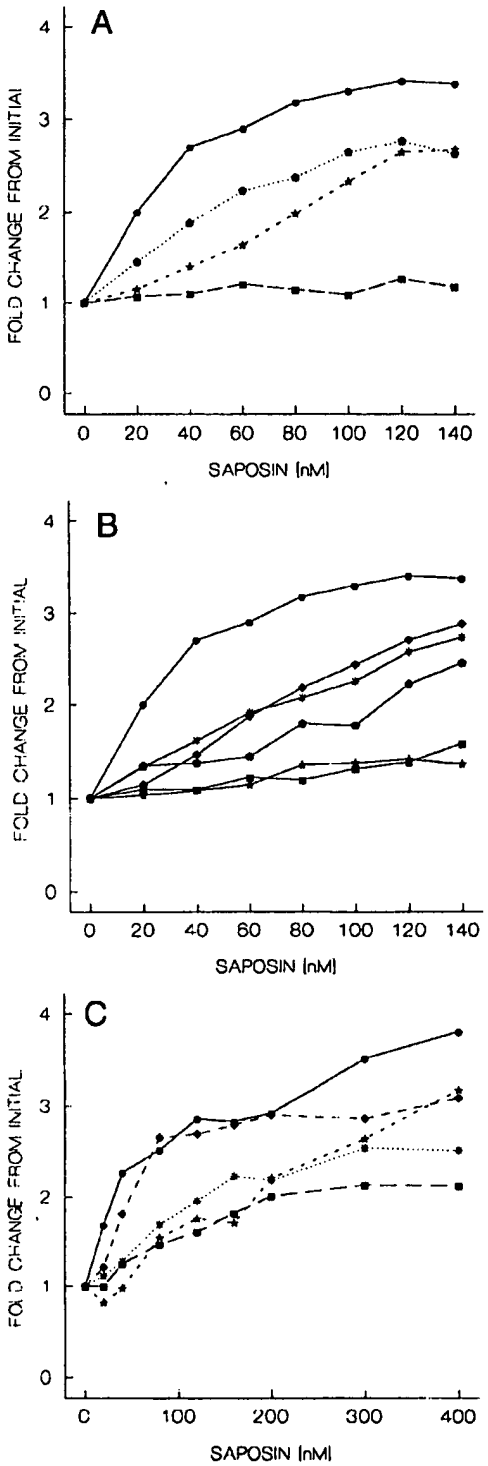

FIG. 4. **Activation profiles for acid β-glucosidase by chimeric (*A*), point-substituted (*B*), or cysteine-mutated (*C*) saposin Cs.** All assays were conducted in the presence of 2 nM acid β-glucosidase, 0.4 μg/ml phosphatidylserine, and 0.1 M sodium acetate, pH 4.7. Enzyme was preincubated with phosphatidylserine and saposin C before substrate was added to start the reaction. The fold change refers to the ratio of final to initial activity. The concentration of the saposin was based on the concentration of the homogeneous protein and the determined or calculated molecular weight. In *A*, saposin C (●), saposin B(1–41)C(42–80) ( ), saposin C(1–41)MC(42–80) (*), and saposin C(1–41)B(42–80) (■) are shown. In *B*, saposin C (●), saposin C Q48W (*), Q48R ( ), Q48N (*), Q48I (♦), and Q48A/E49A (■) are shown. In *C*, the typical effects of cysteine mutated sapsosin Cs are shown. The results are shown for wild-type saposin C (●), C72G (♦), C47S (■), C78R (*), and C78S (*).

Using Mutagenesis—The chimeric saposins and point-mutated saposin Cs are shown in Fig. 2. Saposin B was selected as the "backbone" for the chimeric saposins because it does not interact with acid β-glucosidase (15). Saposins B and C have the same cysteine placement and disulfide bond structure (10), suggesting the potential for highly similar folding and disulfide structure in the wild-type and chimeric saposins. HPLC analyses of Ni^{2+} column purified wild-type and mutant saposins revealed several forms of the recombinant proteins with different retention times (data not shown). ES-MS analyses showed these were oxidized and reduced forms of mono- and dimeric saposins (Table I). Some of saposin C(1–41) and C(42–80) were dimers linked by inter chain disulfide bonds. Partially oxidized saposin C from the carboxyl-terminal 70% was present in small amounts when wild-type saposin C cDNA was expressed. The NH_2-terminal sequence of this peptide was MIDNK, whereas that from the same region of saposin C was TKLIDNK. Presumably, this was due to aberrant initiation in this region. By ES-MS the largest amounts of saposins were monomeric, fully oxidized forms with the expected three disulfide bonds (10); these were used for activation studies.

Neither saposin B(1–52)C(53–80) nor saposin C(1–52)B(53–80) enhanced acid β-glucosidase activity (Fig. 2*A*). Saposin C(1–52)B(53–80) or C(1–41)B(42–80) up to 140 nM also did not inhibit wild-type saposin C/acid β-glucosidase interaction (Fig. 2*A*). Saposin C(1–61)B(59–80) activated acid β-glucosidase to the same level as the wild-type saposin C, but the AC_{50} was nearly 10-fold increased, *i.e.* residues 53–61 are important for the binding to but not activation of acid β-glucosidase. Residues 47–50 are strictly conserved in several species (Fig. 1*B*), but the carboxyl-terminal 50% of the mouse and human saposin Cs differ at 19/39 residues. In the region of residues 48–61, 12 of 14 amino acids are identical or highly similar. Using the cross-species saposin C, HC(1–41)MC(42–80), the AC_{50} was about 3–4-fold increased (~70 nM *versus* 15 nM wild-type) and nearly full activation (70–80%) could be achieved. These results provide additional localization to amino acids 47–61 for essential residues for binding to and activation of acid β-glucosidase by saposin C.

Chemically synthesized saposins C(22–31), C(48–55), and C(53–63) did not interact with acid β-glucosidase (Fig. 2*B*). Saposin C(22–31) was used as an internal control because the NH_2-terminal is not important for interaction with acid β-glucosidase. Other controls for nonspecific effects included human angiotensin I, II, and III and bradykinin. Reduced and carboxymethylated wild-type saposin C (15) (Fig. 2*B*) also did not interact with acid β-glucosidase. These findings showed that there is a requirement for higher order structure of saposin C for interaction with acid β-glucosidase.

The importance of the highly conserved Gln^{48} and Glu^{49} residues (Fig. 1*B*) was evaluated by site-directed mutagenesis. Each of the expressed mutant saposins that were available in sufficient amounts provided single sharp peaks on HPLC and were in the fully oxidized forms (Table I). The singly mutated (Q48N) or the doubly mutated Q48A/E49A saposin Cs did not activate the enzyme nor did they compete with wild-type saposin C (Fig. 2). The next series of point substituted saposin Cs were created from the predicted secondary structure (using the Robson algorithm) surrounding the Gln^{48}-Glu^{49} sequence. As shown in Fig. 5*A*, wild-type saposin C is predicted to have a

TABLE I

Mass determination of wild-type and mutant saposins by ES-MS

ES-MS determined molecular mass. Major peaks (>85% of protein) were isolated from HPLC and analyzed. Minor peaks (<15% of protein) from HPLC represented protein forms with different numbers of disulfide bonds with occasional interchain disulfide bonds (data not shown).

Source	Molecular weight			Disulfide bonds (number)
	Calculated	Nonreduced	Reduced	
Wild-type saposin C	11,718.8	11,712.4	11,718.1	3
Wild-type saposin B	12,589.8	12,583.0	12,590.0	3
Saposin B(1–41)C(42–80)	11,501.4	11,494.7	11,501.2	3
Saposin C(1–41)B(42–80)	11,960.4	11,952.7	11,958.9	3
Saposin C(1–61)B(59–80)	13,060.3	13,053.6	13,059.6	3
Saposin C(1–41)	7,536.3	15,066.6	7,536.6	3 (at least one interchain)
Saposin C(42–80)	6,969.5	13,932.0	6,968.8	3 (at least one interchain)
Saposin C(Q48I)	11,703.9	11,698.7	11,703.9	3
Saposin C(C47S)	11,702.8	11,698.7	11,702.4	2
Saposin C(C72G)	11,672.7	11,669.1	11,673.2	2
Saposin C(C78R)	11,771.8	11,767.9	11,771.6	2

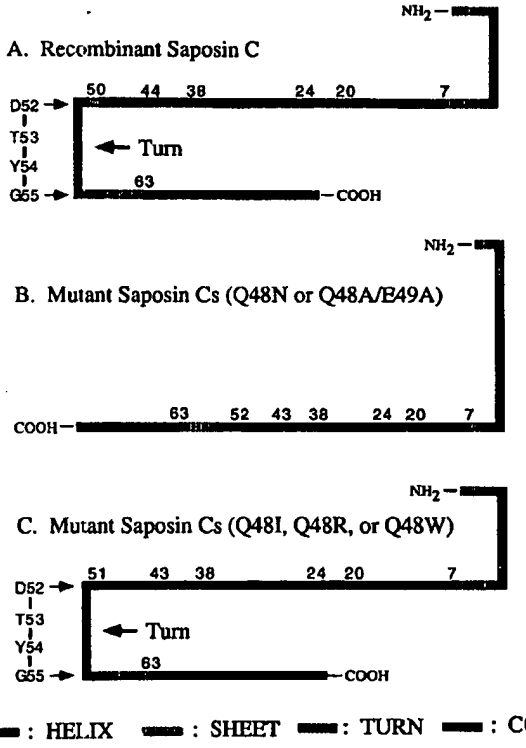

FIG. 5. **Schematic of the predicted effects on secondary structure for wild-type (*A*) or mutant (*B* and *C*) saposin Cs.** The Robson algorithm was used. The boundaries of a predicted helix, sheet, turn, or coil are indicated by the amino acid residue number for ease of orientation. The amino acid numbers refer to those from native saposin C without the additional NH_2- and COOH-terminal sequences of the recombinant saposin Cs. The first turns in *A*, *B*, and *C* include these extra NH_2-terminal amino acids. They have no long range effects on predicted structures. A single major turn is predicted to involve residues 52–55 of the wild-type sequence. In *B* this turn was eliminated by the substitution of Q48N and all other amino acids, except Arg, Trp, and Ile (*C*). Other local secondary structural changes are also predicted between the wild-type (*A*) and point-mutated (*C*) sequences. In the region of the indicated turn, the calculations were not affected by the presence or the absence of the nonsaposin sequences.

major turn near Gln^{48}-Glu^{49} involving residues 52–55 (Fig. 5). By comparison, neither saposin B nor saposin C Q48N or Q48A/E49A have this turn (Fig. 5*B*). We assumed that this predicted turn was important for saposin C-acid β-glucosidase interaction. We modeled all possible substitutions at Gln^{48} and only the highly nonconservative substitutions, Trp, Arg, and Ile were predicted to preserve the turn (Fig. 5*C*). Similar studies for amino acid substitutions at residues 47–55 showed that Gln^{48} was the least tolerant of change for preservation of the turn; *i.e.* 10–15 different substitutions were tolerated at residues other than Gln^{48}. On this basis, saposin Cs $Gln^{48} \rightarrow$ Trp, $Gln^{48} \rightarrow$ Arg, and $Gln^{48} \rightarrow$ Ile were expressed and evaluated. At equimolar amounts these saposins achieved about 70% of the wild-type saposin C stimulation of the enzyme (Fig. 4*B*). The AC_{50} values were 2.5-fold (Gln → Trp and Gln → Ile) to 4-fold (Gln→R) increased. These results were similar to those with the saposins B(1–41)C(42–80) and HC(1–41)MC(42–80), both of which retain the predicted turn. These results indicate that the maintenance of the predicted turn is critical to the functional interaction of saposin C and acid β-glucosidase but that other minor secondary structural changes and/or the specific residues within the Gln^{48}-Glu^{49} region alter the affinity of saposin C for the enzyme and ability to enhance acid β-glucosidase activity.

Singly substituted saposin Cs with Arg or Ser at position 47 or 78 were used to examine the role of disulfide bonds in the effects of saposin C on acid β-glucosidase. By ES-MS analyses each of these cysteine-mutated saposin Cs had two disulfide bonds (Table I). Although activation (2.5–3-fold) of acid β-glucosidase was retained, this was achieved only at concentrations greater than 150 nM (see Fig. 4*C* for typical results). These properties were retained after heating these saposins at 95 °C for 10 min. With the soluble forms of C72G or C72F saposin C, activation was essentially the same as achieved with the wild-type saposin C (Fig. 4*C*). Previously, the C72F or C72G saposins were used directly as Ni^{2+} column purified preparations, and we observed inhibition of wild-type saposin Cs, effects. This was reproducible but only with the preparations containing a large amount of an aggregate. The nonaggregated forms of C72G or C72F activated acid β-glucosidase, by native polyacrylamide gel electrophoresis were monomeric, and had the masses as shown in Table I.

DISCUSSION

Saposin C was first isolated as a heat stable factor from the spleens of Gaucher disease patients (4). This 80-amino acid peptide was thought to function simply as a co-factor for enhancing acid β-glucosidase cleavage of its substrates, glucosylceramide and synthetic β-glucosides (15, 24–27), by binding to the enzyme (13, 28). This physiologic role was confirmed by the presence of a Gaucher disease-like phenotype and glucosylceramide storage in patients with deficient saposin C (29–32). Phosphatidylserine modified the effect of saposin C because low or high concentrations increased or diminished enhancement of activity, respectively (13, 15). Qi *et al.* (15) showed that there is highly specific (nanomolar) interaction of saposin C

with acid β-glucosidase and showed the importance of a folded structure and the carboxyl-terminal 50% for this specific effect. Recently, saposin C was shown to have neurotrophic effects when placed in the medium of neuroblastoma cell lines (17, 18). The current investigations showed the segregation of the different functions of saposin C to discrete regions of the protein.

Previously, we showed that recombinant saposin Cs produced in *E. coli* had properties nearly identical to those of the natural wild-type protein (15). Importantly, for the present studies, HPLC and ES-MS analyses provided insight into the structure of the expressed saposins from *E. coli*. This was important because we had assumed that the conservation of cysteine placement in human and mouse saposins B and C would preserve the formation of disulfide bonds in chimeric saposins composed of components of B and C. In all of the mutant saposins containing the complete sequence and not mutagenized at the cysteines, small yields of saposins with aberrant disulfide structure were detected by HPLC and ES-MS. The isolation and use of fully oxidized saposin C allowed direct comparisons of the properties of wild-type and mutant proteins. Introduction of mutations at cysteines 47 and 78 or cysteine 72 led to a large proportion of the mutant saposins being expressed in inclusion bodies or as aggregates, respectively. This required direct HPLC purification from TFA-solubilized pellets of the Cys^{47} and Cys^{78} mutants. These soluble mutant saposins retained two disulfide bonds. Similarly, monomeric C47G or C47F had normal activation properties, but these proteins aggregated easily. The cysteine-substituted (Arg or Ser) saposin Cs were heated at 95 °C and retained their abnormal acid β-glucosidase activating properties, even though one disulfide bond was disrupted. These results suggest that not all of the disulfide bonds are necessary for the retention of thermal stability properties as assessed in this manner. Our previous work showed that reduction and carboxymethylation destroyed the activating and binding properties of wild-type saposin C (15). This result was confirmed (16). Taken together, these results indicate the need for some disulfide structure for retention of these properties. The fact that the Arg- (nonconservative) or Ser-substituted (conservative and isosteric) saposin Cs at position 47 or 78 had lost their high affinity interaction with acid β-glucosidase indicates the importance of the disulfide bonds rather than the nature amino acid at these residues alone.

The activation effects of saposin C on acid β-glucosidase were regionally localized to the carboxyl-terminal 50% using chimeric and point-mutated proteins. The inactivity of saposin C(1–52)B(53–80) and the activity of saposin C(1–61)B(59–80) as well as the high conservation of residues 47–53 (Fig. 1*B*) indicated the importance of the region spanning amino acids 47–61. Saposin C (Q48N) had an isofunctional substitution but was inactive. Saposin Cs Q48W, Q48R, and Q48I retain activity even though these substitutions are highly nonconservative and disruptive (33). However, only Trp, Ile, and Arg substitutions were predicted to preserve the turn involving residues 52–55. These mutants did retain the activation and binding properties of saposin C but with decreased apparent affinity for the enzyme. The similarity of the results obtained with of the human/mouse saposin C/C and human/human chimeric saposin B/Cs showed that the composition of the amino acids within the region of 43–59 and the structure assumed by the folded molecule were equally important to retention of this property. Apparently, this structure is, at least in part, dictated by a consensus sequence spanning amino acids 47–63 (Fig. 1*B*). This consensus was derived from the four saposin Cs that have

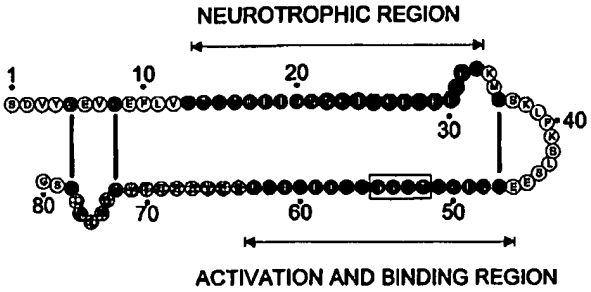

FIG. 6. **A schematic of the functional organization of neurotrophic and acid β-glucosidase activation properties of saposin C.** Except for the *box* that indicates the predicted turn in Fig. 5*A* and the disulfide bonds, the figure is not meant to represent known physical structure. The *darkly highlighted* residues from 13–33 encompass the neurotrophic region. The *heavily circled* residues, 22–32, are of major significance to this effect. The minimal peptide may be as small as residues, 22–29. The region spanning residues 47–63 is important to the acid β-glucosidase activation effects of saposin C, and the presence of all three disulfide bonds is also important for this interaction. Higher order structure is also required to have full activation effects on acid β-glucosidase.

been shown to activate human acid β-glucosidase (9, 34–36).[2]

The conservation of cysteine placement in the saposins has suggested that the disulfide structure of saposin C is essential for the activation of acid β-glucosidase by saposin C (10). Our present results suggest otherwise. The major effect of Arg or Ser substitution at cysteine 47 or 78 was to greatly increase the AC_{50} values with minor effects on the maximal levels of stimulation. This indicates the need for at most two disulfide bonds in saposin C for retention of activity. Furthermore, secondary structure in the carboxyl-terminal 50% of saposin C is important but apparently not sufficient for specific interaction with acid β-glucosidase.

Recently, Weiler *et al.* (16) used chemically synthesized saposin C peptides to suggest that the saposin C activation effects toward acid β-glucosidase are spread throughout the entire molecule. Those results implied that the disulfide structure was not necessary for the activation properties of saposin C. Weiler *et al.* (16) obtained AC_{50} values 3–6 orders of magnitude greater than ours and those extrapolated from the data of others (13, 15). We could achieve only low levels of saposin C effects at μM amounts using their assay system. In addition, the lack of nonsaposin C peptide controls in the studies of Weiler *et al.* (16) makes it difficult to exclude nonspecific interactions between their synthetic peptides and the hydrophobic acid β-glucosidase. Our findings of: 1) highly selective regional localization of activation effects to the carboxyl-terminal 50% of saposin C, 2) the clear lack of effect of the NH_2-terminal 50%, and 3) the inability of various saposin C and nonsaposin C peptides to activate acid β-glucosidase indicate that a much more specific interaction was examined in the present studies.

The localization of the neurotrophic effects to the NH_2-terminal 50% of saposin C is consistent with the recent results of O'Brien *et al.* (17, 18). Indeed, the neurotrophic effect of prosaposin was sublocalized to the saposin C residues 13–29, and 18–29 were suggested as essential. Our studies are consistent with that suggestion because we found that residues 22–31 retain neurotrophic properties. This result refines the localization to residues 22–29. Indeed, on an equimolar basis, our saposin C(22–31) provided similar degrees of neurite outgrowth as did the comparable saposins C(8–29) or C(18–34) of O'Brien *et al.* (18). These results indicate that a linear 7–11 residue sequence is sufficient for the neurotrophic effects of

[2] Y. Sun and G. A. Grabowski, unpublished observations.

saposin C and prosaposin but that longer sequences from the NH_2-terminal half of saposin C enhance the interaction with NS20Y cells; *i.e.* probably increase the affinity for the receptor. The identical effects of the natural (glycosylated) and recombinant (unglycosylated) saposin Cs show that the enhanced effect of longer sequences is not due to the oligosaccharide moiety. Importantly, the neurite outgrowth effects are cell-specific because NS20Y, a cholinergic neuroblastoma cell line, responded better than Neuro2A, a noncholinergic cell line (23). This is consistent with the localization of prosaposin, as opposed to the individual saposins, to cholinergic neurons in rat brain (37). These results imply that the prosaposin-mediated neurotrophic effects, via its saposin C segment, may be mainly in specific sets of neurons.

The segregation of neurotrophic and activation regions of saposin C to the NH_2-terminal and carboxyl-terminal 50% regions, respectively, expands the repertoire of prosaposin functions and its functional organization. The schematic presented in Fig. 6 suggests that these different functions occupy opposite faces of the saposin C molecule. Prosaposin does not substantially activate acid β-glucosidase (15), but it does have neurotrophic effects (17).[3] These results suggest that occupancy of the *N*-glycosylation site of saposin C may keep its NH_2-terminal region exposed and that the carboxyl-terminal region is hydrophobic and buried in prosaposin. This could provide an interesting topographic mechanism for controlling acid β-glucosidase activity during transit through nonlysosomal compartments, *i.e.* acid β-glucosidase would be active only in the lysosome where saposin C is proteolytically liberated from prosaposin (38, 39). In addition, the temporal and spatial regulation of prosaposin expression throughout development (40) and its tissue specific processing indicate that this multifunctional locus encodes biologic properties that extend beyond its initial role as a lysosomal hydrolase activator precursor.

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